The polymorphisms of bovine cocaine- and amphetamine-regulated transcripts and their associations with cattle (*Bos taurus*) growth traits

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We investigated the polymorphisms of bovine cocaine- and amphetamine-regulated transcripts (*CART*). The coding and regulating regions of *CART* were screened in 7 cattle breeds by the single-stranded conformation polymorphism (SSCP) technique. The four loci (C1, C2, C3 and C4) studied were all polymorphic. Polymerase chain reaction (PCR) products representing different SSCP variants were sequenced and a total of 9 single-nucleotide polymorphisms (SNPs) were found. The associations between polymorphic loci and the growth traits of Nanyang cattle were analysed. The results indicated that genotype A_1A_1 of the C1 locus was associated with a higher body weight ($P < 0.05$) than heterozygous A_1B_1. Genotype A_2A_2 of the C2 locus was associated with lower body weight and average daily weight gain ($P \leq 0.001$) than heterozygous A_2B_2. C3 and C4 loci had no significant effect on Nanyang cattle growth traits ($P > 0.05$).

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1. Introduction

Cocaine- and amphetamine-regulated transcript (CART) peptides are neurotransmitters that have recently been the focus of much attention as mediators of feeding behaviour and body weight regulation in mammals. CART peptides were found and sequenced long ago, and the mRNA encoding the peptides was detected in 1995 (Douglass *et al* 1995). CART peptides were found to be significant regulators of body weight via both feeding behaviour and control of digestion and metabolism (Okumura *et al* 2000; Aja *et al* 2001; Wortley *et al* 2004). *CART* is highly expressed in the arcuate and paraventricular nuclei of the hypothalamus, areas known to be involved in the control of appetite and energy expenditure (Douglass *et al* 1995; Koylu *et al* 1997; Elias *et al* 1998). Hypothalamic *CART* mRNA levels are decreased in hypoleptinaemic states, such as prolonged fasting or in the leptin-deficient ob/ob mouse (Kristensen *et al* 1998). Moreover, *CART* knocked-out mice displayed increased body weight at the age of 40 weeks, without any difference in food intake (Wierup *et al* 2005). A missense mutation in *CART*, which changes Leu at codon 34 of proCART to Phe, has been discovered in a 10-year-old Italian boy who has been obese since the age of 2 years (Giudice *et al* 2001). This indicates that *CART* plays a role in energy utilization. Interestingly, *CART* expression in the arcuate nucleus of Siberian hamsters was consistently upregulated during short photoperiods in both genders

Keywords. *CART*; cattle; growth traits; SNPs

Abbreviations used: CART, cocaine- and amphetamine-regulated transcripts; LSM, least square means; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; SSCP, single-stranded conformation polymorphism; TBE, Tris-borate-EDTA

(Adam *et al* 2000; Mercer *et al* 2001). *CART* may therefore have a role in driving seasonal appetite change.

In ruminants, regulatory mechanisms for certain types of food intake have been considered to be more important than others. For beef cattle, food intake is significantly associated with growth rate and body weight. A few studies have been done to investigate the genetic mechanism of cattle food intake. Research on the molecular genetic mechanisms of human and model animals indicated that *CART* plays an important role in regulating food intake and body weight. Hence, *CART* may have a similar function in cattle. Some single-nucleotide polymorphisms (SNPs) of bovine *CART* were detected, but their associations were not analysed (Valle *et al* 2004). Taken together, the objective of this study was to screen the SNPs in the coding and flanking region of *CART* and to examine the association of *CART* polymorphisms with performance traits in 7 cattle breeds.

2. Materials and methods

2.1 *Sampling and DNA extraction*

In this study, 613 female cattle belonging to the Nanyang (240), Qinchuan (68), Jiaxian Red (146), Jinnan (43), Luxi (19), Angus (36) and Chinese Holstein (61) breeds were included. Nanyang, Qinchuan, Jiaxian Red, Jinnan, Luxi are five important breeds for beef production in China, while Angus is a beef breed introduced from Canada to China. Chinese Holstein is a dairy breed. Blood samples were obtained from these cattle populations and genomic DNA was extracted from the blood samples using standard methods. Nanyang cattle were fed on an *ad libitum* concentrate and straw for up to 24 months of age, after weaning at 6 months of age. The live weight and average daily weight gain of Nanyang were determined at the age of 6 and 24 months.

2.2 *Primer design and polymerase chain reaction amplification*

Four pairs of polymerase chain reaction (PCR) primers (table 1) were designed to amplify the coding and flanking regions of bovine *CART* (GenBank accession number NW_929854) using the Primer V5.0 software. The PCR products covered almost the entire sequences of *CART*, so all the polymorphisms of this gene could be detected. The 20 μl PCR reaction volume contained 50 ng DNA template, 0.20 mM dNTP, 2.5 mM $MgCl_2$ and 0.5 U *Taq* DNA polymerase (TaKaRa, Dalian, China). The PCR protocol was performed as per the following programme: 94°C for 5 min followed by 35 cycles at 94°C for 40 s, annealing for 40 s, and 72°C for 1 min and a final extension at 72°C for 10 min.

2.3 *Single-stranded conformation polymorphism (SSCP)*

Aliquots of 5 μl of the above PCR products were mixed with 5 μl of the denaturing solution (95% formamide, 25 mM EDTA, 0.025% xylene–cyanole and 0.025% bromophenol blue), heated for 10 min at 98°C and chilled on ice. Denatured DNA was subjected to 10% PAGE (80×73×0.75 mm) analysis which was run with 1× Tris-borate-EDTA (TBE) buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) for 2 h at room temperature under a constant voltage (150 V). The gel was stained with silver nitrate and visualized with 2% NaOH solution (containing 0.1% formaldehyde) according to Zhang *et al* (2007).

2.4 *DNA sequencing analysis*

The PCR products from different PCR-SSCP genotypes were purified with the GenElute PCR DNA Purification Kit (Sigma-Aldrich Corporation, USA) and sequenced by the ABI 377 sequencer from both directions (Applied Biosystems, USA). Sequences were aligned using the

Table 1. Primers used for polymerase chain reaction (PCR) amplification

Locus	Position (Ref. NW_929854)	Sequence	Temperature (°C)
C1	328702–329430	5′ TTTGTAAGGGTAGAGCCAGGAC 3′ 5′ CTGAAACTCGGCGCTACTCG 3′	63
C2	329418–329907	5′ CGCCGAGTTTCAGCACCATG 3′ 5′ CCTGGACTCCGCAAAGAGGG 3′	66
C3	329836–330351	5′ CCTGCGGTCAGTACGTGGGA 3′ 5′ CACAGAAACCCAAGGGCAGA 3′	66
C4	330524–331147	5′ TCCTGTCTGTTCACCGTCCTT 3′ 5′ A/AGCCAGACTCCAGGGATGA 3′	67

Note: Exons 1, 2 and 3 are located at base pairs 329435–329593, 330019–330102, 330965–331072, respectively (GenBank accession No. NW_929854).

web-based CLUSTAL-W (*http://www.ebi.ac.uk/clustalw/index.html*) program. In a specific locus, the PCR products that represented different PCR-SSCP genotypes were sequenced, so several SNPs may be found in a single PCR product. Mutations appeared simultaneously in the individuals with specific PCR-SSCP genotypes.

2.5 *Statistical analysis*

Allelic frequencies were determined by direct counting for each breed. Statistical analysis was performed on records of growth traits in Nanyang heifers (N = 240). All the association analyses were done in two steps, first using a full animal model and then using a reduced animal model. The full animal model included fixed effects of marker genotype, birth year, season of birth (spring vs fall), age of dam, sire, farm, sex and random effects of animal. The reduced model (Henderson 1986) was used in the final analysis. The software SPSS (version 13.0) was used to analyse the relationship between the genotypes and traits in cattle. The adjusted linear model with fixed effects was established including the effects of sire, dam within sire, age and genotype, as well as interaction between sire and genotype. Adjusted linear model: $Y_{ijklm} = \mu + S_i + D_{ij} + A_k + G_l + (SG)_{il} + e_{ijklm}$, where Y_{ijklm} was the trait measured in each of the $_{ijklm}$th animal, μ was the overall population mean, S_i was the fixed effect associated with sire i, D_{ji} was the fixed effect associated with dam j and sire i, A_k was the fixed effect due to the $_k$th age, G_1 was the fixed effect associated with the $_l$th genotype, $(SG)_{il}$ was the interaction between the sire I and genotype 1, and e_{jiklm} was the random error. The effects associated with farm, sex and season of birth (spring vs fall) were not matched in the linear model, as the preliminary statistical analysis indicated that these effects did not have a significant influence on the variability of traits in the analysed populations. The least square means (LSM) estimates with standard errors for two *CART* genotypes and growth traits were analysed.

3. Results and discussion

SSCP polymorphisms were detected in each of the four loci of *CART*. The number of bands and their positions in the gel clearly showed the occurrence of DNA sequence variations. The PCR products of different SSCP variants were sequenced and 9 SNPs were found. Based on SSCP and responsive sequence variations, the genotype and allelic frequencies were analysed (table 2). In the C1 locus, two SSCP genotypes were identified and named as genotypes A_1A_1 and A_1B_1. Genotype A_1A_1 was more prevalent, no genotype A_1B_1 was detected in the Jinnan and Angus breeds. All the breeds were in Hardy–Weinberg equilibrium at the C1 locus. The sequencing analysis of the two genotypes revealed four SNPs g.[–636T>C; –521T>C; –431T>C; –398T>C]. They formed two consistent haplotypes (A_1, B_1). The frequencies of genotype A_1B_1 and allele B_1 were higher in Holstein than in the other breeds. In the C2 locus, three SSCP genotypes were identified and named as genotypes A_2A_2, A_2B_2 and B_2B_2. All the breeds were in Hardy–Weinberg equilibrium at the C2 locus except Chinese Holstein. Genotype A_2A_2 was more prevalent, and genotype B_2B_2 was not detected in the Angus breed. The sequence analysis of the three genotypes revealed an SNP (g.234A>G). This SNP has been detected before (Valle *et al* 2004). The frequency of genotype B_2B_2 was higher in Luxi and Jinnan than in the other breeds. Interestingly, the frequency of genotype A_2B_2 was significantly higher in the Holstein breed than in the other breeds. This may be due to the specific selection for milk production that affected the frequency directly or indirectly. In the C3 locus, three SSCP genotypes were identified and denoted as genotypes A_3A_3, A_3B_3 and B_3B_3. All the breeds were in Hardy–Weinberg equilibrium at the C3 locus except Qinchuan and Jiaxian. Genotype A_3A_3 was more prevalent; genotype B_3B_3 was only detected in the Qinchuan and Jiaxian breeds. The sequence analysis of the three genotypes revealed two SNPs g.[707 G>C; 782G>A]. They formed two consistent haplotypes (A_3, B_3). The frequencies of genotype A_3B_3 and allele B_3 were higher in Luxi, Jiaxian and Holstein than in the other breeds. In the C4 locus, two SSCP genotypes were identified and denoted as genotypes A_4A_4 and A_4B_4. All the breeds were in Hardy–Weinberg equilibrium at the C4 locus. Genotype A_4A_4 was more prevalent, genotype A_4B_4 was detected only in the Qinchuan, Nanyang and Jiaxian breeds. The sequence analysis of the two genotypes revealed two SNPs g.[1418C>T;1420C>G]. They formed two consistent haplotypes (A_4, B_4). However, we did not find significant linkage disequilibrium between the four loci.

In present study, the short (200–300 bp) fragments of bovine CART were difficult to amplify by PCR because of the base composition, so the large fragments were used for SSCP analysis. Some SNPs may not have been identified, as the fragments were rather large (489–728 bp) for SSCP analysis and the primer sites overlapped with the beginning of some exons.

All the SNPs detected in present study were located at the non-coding region of *CART*. This was similar to the findings of others (Yamada *et al* 2002; Valle *et al* 2004), which suggested that *CART* was functionally important. Unlike the coding regions, the putative promoter and intron regions were highly polymorphic. Four SNPs were detected in the 5′ flanking region of bovine *CART* (C1 locus) by PCR-SSCP and DNA sequencing. The mouse *CART* promoter sequence has been characterized and many transcription-binding sites have been identified (Dominguez and Kuhar 2004).

Table 2. Genotype and allelic frequencies of different polymorphic loci in the 7 cattle populations

Locus			Breeds						
			Qinchuan	Nanyang	Jiaxian	Jinnan	Luxi	Angus	Holstein
C1	Genotype	A_1A_1	0.90	0.85	0.98	1.00	0.95	1.00	0.76
		A_1B_1	0.10	0.15	0.02	0.00	0.05	0.00	0.24
	Allele	A_1	0.95	0.93	0.99	1.00	0.97	1.00	0.88
		B_1	0.05	0.07	0.01	0.00	0.03	0.00	0.12
Standard errors of gene frequencies			<0.0001	<0.0005	<0.0001	0	0.0008	0	0.0008
C2	Genotype	A_2A_2	0.76	0.66	0.82	0.55	0.52	0.75	0.27
		A_2B_2	0.21	0.31	0.17	0.30	0.24	0.25	0.71
		B_2B_2	0.03	0.03	0.01	0.15	0.24	0.00	0.02
	Allele	A_2	0.87	0.81	0.91	0.70	0.65	0.88	0.62
		B_2	0.13	0.19	0.09	0.30	0.35	0.12	0.38
Standard errors of gene frequencies			0.0002	0.0011	0.0003	0.0025	0.0063	0.0015	0.0019
C3	Genotype	A_3A_3	0.92	0.94	0.80	1.00	0.89	1.00	0.86
		A_3B_3	0.04	0.06	0.16	0.00	0.11	0.00	0.14
		B_3B_3	0.04	0.00	0.04	0.00	0.00	0.00	0.00
	Allele	A_3	0.93	0.97	0.88	1.00	0.95	1.00	0.93
		B_3	0.07	0.03	0.12	0.00	0.05	0.00	0.07
Standard errors of gene frequencies			0.0001	0.0002	0.0003	0	0.0013	0	0.0005
C4	Genotype	A_4A_4	0.90	0.82	0.86	1.00	1.00	1.00	1.00
		A_4B_4	0.10	0.18	0.14	0.00	0.00	0.00	0.00
	Allele	A_4	0.95	0.91	0.93	1.00	1.00	1.00	1.00
		B_4	0.05	0.09	0.07	0.00	0.00	0.00	0.00
Standard errors of gene frequencies			<0.0001	0.0006	0.0002	0	0	0	0

It was found that the proximal promoter encompassed more than 340 nucleotides upstream of the site for transcription initiation, and served as an important region for basal and stimuli-induced transcriptional regulation. There is >80% sequence identity between the mouse and bovine sequences, thus the SNPs g.[–636T>C; –521T>C; –431T>C; –398T>C] detected in locus C1 may be located at the putative promoter region of bovine *CART*. These polymorphisms may influence the expression of *CART*. To test if the polymorphisms within *CART* contributed to the variation in energy utilization, we performed an association analysis between *CART* variants and average daily gain in body weight of Nanyang. The results indicated that the body weight of individuals with the genotype A_1A_1 was 7.6% higher ($P<0.05$) than those with genotype A_1B_1 (table 3). Similar results have been reported in humans, where a functional SNP g.–156 T>C was associated with morbid obesity (Yamada *et al* 2002; Dominguez and Kuhar 2004).

The genotype A_2A_2 was associated with lower body weight and average daily weight gain in Nanyang ($P\leq0.001$) than A_2B_2 (table 3). The body weight of individuals with the genotype A_2B_2 was 6.2% higher and the average daily weight gain of individuals with this genotype was 22.0% higher than those with genotype A_2A_2. The SNP in the C2 locus of *CART* was in intron 1. It could not result in the mutation of amino acid. Nevertheless, it was close to the exon1–intron1 junction, where it was important for mRNA splicing (Krawczak *et al* 2006). Functional SNPs in the 5′ flanking region of *CART* have been detected in previous studies and in other organisms (Yamada *et al* 2002; Dominguez and Kuhar 2004). Therefore, the association can be attributed to linkage disequilibrium with some SNPs in the regulating region of *CART*. This needs to be confirmed by future studies.

This was a preliminary study, which indicated that *CART* played an important role in the regulation of cattle food

Table 3. Least square means and standard errors of the growth traits of Nanyang for the locus genotypes in *CART*

Locus	Genotype (number)	Growth traits	
		Body weight (kg)	Average daily gain (g)
	A_1A_1 (204)	369.2±5.4	385±16
C1	A_1B_1 (36)	343.0±3.8	322±26
	P-value	0.02	0.07
	A_2A_2 (158)	355.9±2.7	345±8
C2	A_2B_2 (82)	378.0±3.5	421±18
	P-value	<0.001	0.001
	A_3A_3 (225)	353.6±3.3	363±42
C3	A_3B_3 (15)	349.0±4.0	354±34
	P-value	0.584	0.675
	A_4A_4 (197)	358.4±3.5	358±7
C4	A_4B_4 (43)	343.7±10.1	350±27
	P-value	0.160	0.731

intake and energy homeostasis. The genetic mechanism of cattle food intake and energy homeostasis in bovine breeds should be further investigated by quantitative trait loci and/or linkage disequilibrium mapping techniques for its importance in beef production.

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